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Short communication

Skinned smooth muscle: calcium-calmodulin activation independent of myosin phosphorylation

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ABSTRACT

In chemically skinned chicken gizzard smooth muscle fibers investigated shortly after preparation, a contraction may be induced by calcium and calmodulin which is independent of myosin phosphorylation at intermediate Ca^{2+}-concentrations. However, fibers stored for a prolonged period also contract in the absence of exogenous calmodulin and exhibit a close relationship between force development and myosin phosphorylation.

KEY WORDS: Smooth muscle - Skinned fibers - Calmodulin - Myosin phosphorylation

INTRODUCTION

In smooth muscle as in skeletal muscle, the contractile proteins are activated by increasing the intracellular free calcium ion concentration, but the calcium sensor is calmodulin rather than troponin. Calmodulin is essential for smooth muscle contraction which is said to be activated by calmodulin dependent phosphorylation of the 20,000 dalton myosin light chain by myosin light chain kinase (MLCK)(1, for review). Here we report that in addition calmodulin may also activate smooth muscle contraction by a different pathway which does not involve the myosin light chain kinase dependent phosphorylation of myosin. The proposed new calmodulin dependent activating mechanism appears to be distinct from other calcium dependent and myosin light chain kinase independent activation mechanisms of smooth muscle contractile proteins (2,3,4,5) as these are not dependent on calmodulin.

MATERIALS AND METHODS

Smooth muscle strips from the outer circumferential layer of freshly dissected gizzard obtained immediately after slaughtering the chicken were immersed for 30 min in an ice-cold solution containing (mM): EGTA 5, KCl 50, sucrose 150, imidazole 20 (pH 7.4) and dithioerythrol 2; they were subsequently skinned by the addition of 1% (v/v) Triton X-100 for a further 4 hrs at 4°C (cf. 8, 15). Subsequently the fibers were again immersed into the Triton-free preskinning solution and stored (for less than 1 week) in a solution containing 50% v/v glycerol and 50% relaxing solution pH 7 at -20°C until used. Aged preparations (cf. Table 1b, rows G and H) were obtained after storing the fibers at -20°C for up to 8 weeks. For recording isometric contractions small fiber bundles (about 5 mm in length and 50 to 200 µm in diameter) were glued to an AME 801 force transducer (SensoNor, Horten, Norway) in a nominally calcium-free relaxing solution containing (mM): EGTA 4, KCl 50, $MgCl_2$ 5, imidazole 25, ATP 1, creatine phosphate 1, creatine phosphokinase (Boehringer Mannheim, FRG) 0.4 mg/ml as well as calmodulin at the concentrations indicated. The pH was adjusted to 7.0, T = 20°C. For activation the desired Ca^{2+}-concentration was obtained by adjusting the proportion of EGTA and Ca-EGTA and the free Ca^{2+}-concentration was calculated by using an apparent binding constant of 2 x $10^7 M^{-1}$ (cf. 16). Activating solutions also contained calmodulin from bovine testis (17) in varying concentrations, as indicated in the Table. Maximal contractile tension was 19 N/cm^2 ±3 N/cm^2 (n=5) in 50 µm thick fiber bundles. LC-20 phosphorylation was determined in parallel in other fiber bundles which were subjected to the same experimental protocol except that the fiber bundles were immersed in 15% ice-cold trichloroacetic acid at the desired time (20 min after onset of contraction). Phosphorylated and non-phosphorylated regulatory light chains were quantified by 2D-gelelectrophoresis according to (18) and in some experiments according to (19); myosin light chain satellites were never observed.

RESULTS AND DISCUSSION

To study the activating effects of calmodulin the cell membrane of chicken gizzard smooth muscle was chemically removed by a Triton X-100 skinning procedure, which renders the contractile structures accessible to exogenous calmodulin and other proteins. In such skinned fibers, suspended in ATP salt solution (see legend to Table 1), contraction-relaxation cycles could be elicited by raising and lowering the free calcium ion concentration in the bathing medium. Freshly prepared skinned fibers of chicken gizzard were fully relaxed at pCa 5.8 if exogenous calmodulin was not present while the extent of myosin phosphorylation was low (cf. Table 1, row B). At pCa 5.8 addition of 0.05 µM calmodulin, however, caused a pronounced contraction (75% maximal) without increase in the extent of light chain-20 phosphorylation (Table 1, rows C, D). Maximal contraction (19 N/cm^2) was obtained at pCa 5.2 with 3 µM calmodulin which increased the extent of light chain phosphorylation to a level of nearly 50%.

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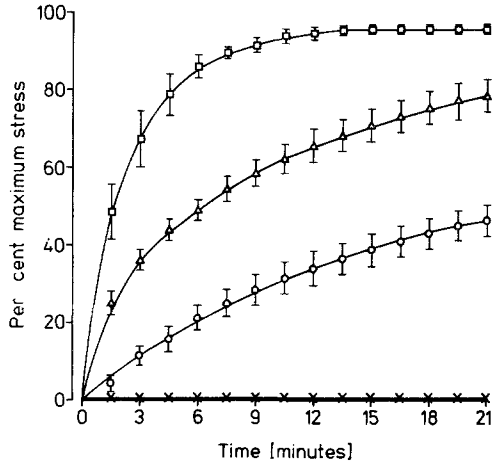
FIGURE 1



Calmodulin activation of skinned chicken gizzard. Time course of force development of skinned fibers from chicken gizzard at constant pCa 5.8 in the absence (x) and in the presence of increasing concentrations of calmodulin (CaM): 0.05 μM (O), 0.1 μM (Δ) and 0.5 μM (□). For composition of solutions cf. Materials and Methods. n= 5-7 fiber bundles for each CaM concentration

As shown in Figure 1, not only the extent, but also the rate of force development was calmodulin-dependent. This slow activation was not due to diffusional limitations, since the skinned fibers were preincubated with calmodulin in relaxing solution for prolonged periods (approximately 15 min) prior to adjusting the pCa to 5.8. The contraction which could be induced by calmodulin and pCa 5.8 at basal levels of myosin phosphorylation was abolished by trifluoperazine (10^{-4} M) and could be restored by addition of 3 μM calmodulin. To obtain further evidence for calcium-calmodulin activation of contraction without an increase in myosin phosphorylation the substrate ATP was replaced by ITP which, like CTP, is no substrate for MLCK, but can be used by the contractile system (cf. 6). In contrast to skinned guinea pig taenia coli (21), in skinned fibers of chicken gizzard dephosphorylated in nucleotide-free salt solutions addition of Mg^{2+}-ITP (2 mM) elicited a contraction reaching 37,8±2,1% (n=8) of the maximal force obtained with Mg^{2+}-ATP as substrate at pCa 5.2 and 3 μM calmodulin. No force development occurred with ITP at pCa 5.2 in the absence of exogenous calmodulin.

In conclusion, low concentrations of calcium and calmodulin may activate smooth muscle of freshly prepared skinned gizzard fibers (stored for less than 1 week in glycerol) to produce over 70% of maximal force without noticeable increase in the extent of myosin phosphorylation. On the contrary, phosphorylation-dependent contractions were found in skinned fibers which were aged by prolonged (more than 6 weeks) storing at -20°C in glycerol containing relaxing solution. For reasons which are not yet understood the storage of these fibers changed the force-phosphorylation relationship progressively. After about 8 weeks storage they were still relaxed at pCa 8 but produced 62% of maximal tension at pCa 5.8 even in the absence of exogenous calmodulin, while the extent of myosin phosphorylation rose to 42% (cf. Table 1b). Maximal force development occurred at pCa 5.2 in the presence or absence of exogenous calmodulin.

TABLE 1

Effect of calmodulin (CaM) on force development and myosin light chain phosphorylation in freshly skinned fibres (1a) and aged fibers (1b)

1a	pCa	CaM (μM)	Force	(%)	P-LC-20	(%)
A	8	0	0	(6)	8.7±1.4	(6)
B	5.8	0	0	(6)	8.3±0.6	(6)
C	5.8	0.05	85.7±3.2	(7)	8.7±1.1	(7)
D	5.8	0.1	89.9±3.2	(6)	11.5±0.9	(6)
E	5.8	0.5	96.1±0.8	(5)	29.6±1.4	(5)
F	5.2	5	120.7±2.3	(5)	47.8±1.2	(6)

1b	pCa	CaM (μM)	Force (%)		% LC-phosphorylation	
G (aged)	5.8	0	62.4±4.9	(5)	42.6±2.6	(5)
H (aged)	5.2	0	100		48.2±2.3	(5)

Note the variable relation between myosin light chain-20 phosphorylation (in % of total LC 20) and developed force (given as % of force at pCa 5.2 in the absence of exogenous calmodulin). At pCa 8 skinned gizzard fibers are relaxed and the phosphorylation of myosin is basal (0.1 μmole phosphate/mole light chain), even in the presence of 0.5 μM exogenous calmodulin. If the Ca^{2+}-concentration is increased to pCa 5.8, there is still no force development and phosphorylation remains at basal levels (cf. rows A and B) provided that no exogenous calmodulin is added. Addition of calmodulin (0.05 or 0.1 μM) does not increase the extent of light chain phosphorylation, but induces over 70% of the force (rows C and D) obtained at maximum activation (pCa 5.2, 5 μM calmodulin). Note that contraction induced with higher levels of exogenous CaM and Ca^{2+} is associated with a significant increase in light chain phosphorylation (rows E and F), as was also the case in aged fibers.

Incidentally, these data on aged fibers agree with earlier experiments reporting a calcium-calmodulin induced contraction in various kinds of skinned smooth muscle including chicken gizzard, which were found to be myosin phosphorylation dependent (6,7,9,10,11). We, therefore, propose two mechanisms by which the calcium-calmodulin complex may activate smooth muscle contraction, one involving activation of MLCK and phosphorylation of a myosin regulatory light chain, and the other one bypassing the MLCK-dependent activating system. The latter calmodulin-dependent regulatory mechanism supports considerable force development without increased myosin phosphorylation and may also be involved in stress maintenance at low LC-20 phosphorylation in skinned (11) and intact (20) preparations of smooth muscle. It will be intriguing to find out whether this new mechanism is thin filament linked (12) and depends on the interaction of calmodulin with caldesmon (13,14), which has also been implicated in the regulation of smooth muscle and non-muscle motility.

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REFERENCES

1. Hartshorne DJ, Mrwa U (1982) Regulation of smooth muscle actomyosin. Blood Vessels 19:1-18

2. Persechini A, Mrwa U, Hartshorne DJ (1981) Effect of phosphorylation on the actin-activated ATPase activity of myosin. Biochem Biophys Res Commun 98:800-805

3. Cole HA, Grand RJA, Perry SV (1982) Non-correlation of phosphorylation of the P-light chain and the actin activation of the ATPase of chicken gizzard myosin. Biochem J 206: 319-328

4. Ebashi S (1980) Regulation of muscle contraction. Proc R Soc (London) B 207:259-286

5. Kaminski EA, Chacko S (1984) Effects of Ca^{2+} and Mg^{2+} on the actin-activated ATP hydrolysis by phosphorylated heavy meromyosin from arterial smooth muscle. J Biol Chem 259: 9104-9108

6. Cassidy P, Hoar PE, Kerrick WGL (1979) Irreversible thiophosphorylation and activation of tension in functionally skinned rabbit ileum strips by ^{35}S ATP-γ-S. J Biol Chem 254:11148-11153

7. Hoar PE, Pato MD, Kerrick WGL (1985) Myosin light chain phosphatase. J Biol Chem 260:8760-8764

8. Wagner J (1985) Calmodulin-induced contraction of "native" skinned chicken gizzard is not associated with phosphorylation of light chain-1. J Muscle Res Cell Mot 6:123

9. Cassidy PS, Kerrick WGL, Hoar PE, Malencik DA (1981) Exogenous calmodulin increases Ca^{2+} sensitivity of isometric tension activation and myosin phosphorylation in skinned smooth muscle. Pflügers Arch 392:115-120

10. Gagelmann M, Mrwa U, Pfitzer G, Troschka M, Obst C, Herrmann R, Rüegg JC (1982) Comparison of force and myosin light chain phosphorylation in skinned smooth muscle fibers from chicken gizzard. J Muscle Res Cell Mot 3:478

11. Chatterjee M, Murphy RA (1983) Calcium-dependent stress maintenance without myosin phosphorylation in skinned smooth muscle. Science 221:464-466

12. Marston SB, Smith CWJ (1985) The thin filaments of smooth muscle. J Muscle Res Cell Mot 6:669-708

13. Sobue K, Muramoto K, Inui M, Kanada K, Kakiuchi S (1981) Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. Proc Natl Acad Sci USA 78:5652-5655

14. Ngai PK, Walsh MP (1985) Properties of caldesmon isolated from chicken gizzard. Biochem J 230:695-707

15. Sparrow MP, Mrwa U, Hofmann F, Rüegg JC (1981) Calmodulin is essential for smooth muscle contraction. FEBS Lett 125:141-145

16. Portzehl M, Caldwell PC, Rüegg JC (1964) The dependence of contraction and relaxation of muscle fibres from the crab Maia squinado on the internal concentration of free Ca^{2+}-ions. Biochim Biophys Acta 79:581-591

17. Gopalakrishna R, Anderson WB (1982) Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenylsepharose affinity chromatography. Biochem Biophys Res Commun 104:830-836

18. Gagelmann M, Rüegg JC, DiSalvo J (1984) Phosphorylation of the myosin light chains and satellite proteins in detergent-skinned arterial smooth muscle. Biochem Biophys Res Commun 120:933

19. Haeberle JR, Hott JW, Hathaway DR (1984) Pseudophosphorylation of the smooth muscle myosin light chain: an artefact due to protein modification. Biochim Biophys Acta 790:78-86

20. Aksoy MO, Murphy RA, Kamm KE (1982) Role of Ca^{2+} and myosin light chain phosphorylation in regulation of smooth muscle. Am J Physiol 242:C109-C116

21. Arner A, Wagner J, Rüegg JC (1985) Ca^{2+}-calmodulin-activation of contraction at stabilized levels of myosin phosphorylation in chemically skinned smooth muscle. Acta Physiol Scand 124 (Suppl 542):210

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